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LACTOBACILLUS TRICHODES NOV. SPEC., A BACTERIUM CAUSING SPOILAGE IN APPETIZER AND DESSERT WINES[1]

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EVIDENCE for the existence of an unusual species of *Lactobacillus* capable of growing in wines of high alcohol content has accumulated during the past two decades. The organism is exacting in its growth requirements. Hence the pure-culture studies carried out independently in Australia and California have not been made until recently.

Douglas and McClung (1937)[5] isolated from California wines a bacterium capable of causing spoilage in the presence of more than 20 per cent of alcohol by volume and commonly occurring as masses of long entangled filaments. These authors described some of the characteristics of the organism, but owing to difficulties experienced in culturing it they did not describe it fully nor determine its systematic position.

Fornachon (1943) isolated a similar organism from Australian wines, described it, and placed it in the heterofermentative group of the genus *Lactobacillus*. He did not give the organism a specific name, but referred to it as *Lactobacillus* Type I.

Subsequent investigations have indicated the identity of the organisms studied by the Californian and Australian workers. As the morphology, physiology, and cultural characters of the organism distinguish it from other species of *Lactobacillus* which have been described, we propose that it be recognized as a species. For this new species we propose the name *Lactobacillus trichodes* because of its marked tendency to grow as long intertwined chains and filaments resembling a mass of hair.[6] The typical morphology of this species is shown in figure 1.

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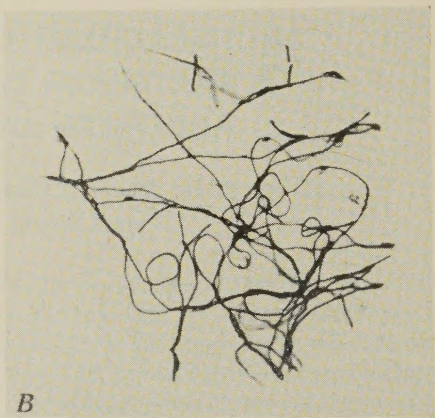
[5] See "Literature Cited" for citations, referred to in the text by author and date.

[6] We are indebted to Dr. R. E. Buchanan for assistance with the nomenclature.

DESCRIPTION OF LACTOBACILLUS TRICHODES NOV. SPEC.

Morphology (at 25° C)

Rods 0.4 to 0.6 by 2 to 4 microns, occurring singly, in pairs and in chains. The organism has a very marked tendency to grow into very long, threadlike chains and filaments, which frequently form a tangled mass. Nonmotile. Without endospores. Gram-positive in young cultures, but in old cultures some cells are Gram-negative.



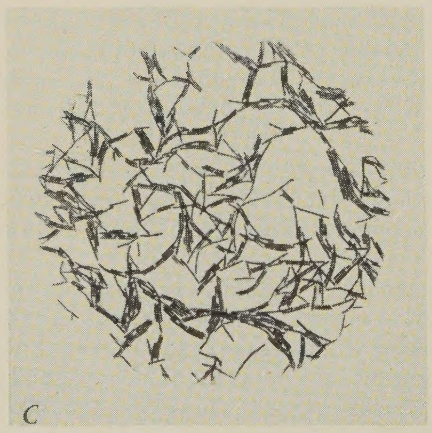


Fig. 1. Morphology of *Lactobacillus trichodes: A,* Deposit from wine infected with *L. trichodes.* Note the beaded appearance of old tangled filaments. *B,* Pure culture of *L. trichodes* showing long tangled filaments. Grown for 16 days at 23° C in autolyzed yeast glucose broth containing 15 per cent of alcohol. *C,* Pure culture of *L. trichodes* showing rods and short filaments after repeated subculture in alcohol-free, autolyzed yeast glucose broth. This culture was incubated 6 days at 23° C. (All × 1,000.)

Cultural Characteristics

Autolyzed yeast glucose gelatin stab: Growth, if any, scant. No liquefaction.

Autolyzed yeast glucose agar slant: Growth, if any, scant.

Autolyzed yeast glucose agar colonies: Colonies develop slowly; are small, subsurface, creamy white, and irregular in shape.

Nutrient agar: No growth.

Potato slant: No growth.

Autolyzed yeast glucose broth: Medium becomes turbid after 3 to 5 days and exhibits a very pronounced silky, wavy appearance when shaken gently.

After 2 to 3 weeks the organism settles and forms a compact sediment. Sometimes the organism grows as a flocculent deposit consisting of long, tangled chains and filaments while the liquid above remains almost clear. No growth in nutrient broth, glucose peptone broth, bacto-yeast extract broth, or grape juice.

Litmus milk: No growth.

Biochemical Characteristics

Catalase: This enzyme is not produced.

Substances fermented: Acid formed in glucose and fructose. Sometimes a little acid formed in sucrose and maltose. Arabinose, xylose, galactose, mannose, lactose, raffinose, glycerol, mannitol, malic acid, citric acid, and tartaric acid are not attacked.

Products of fermentation: Lactic and acetic acids, carbon dioxide, and alcohol are the chief products from glucose; and lactic and acetic acids, carbon dioxide, and mannitol are formed from fructose.

Requirements for Growth

Oxygen relations: Micro-aerophilic.

Optimum temperature range: Optimum range for growth is 25° to 30° C in alcohol-free media and 20° to 25° C in wine or other media of high alcohol content.

pH range: Optimum initial pH range for growth is between 4.5 and 5.5. The organism usually fails to grow when the initial pH of the medium is above 5.8 or below 3.5.

Alcohol tolerance: Grows vigorously in wine containing 20 per cent of alcohol by volume. A few strains grow in 21 per cent alcohol.

Suitable media: Vigorous growth has been obtained only in wine and in media containing yeast autolysate.

Ecology

Sources from which isolated: Dessert and appetizer wines and lees.

Geographical distribution: Widely distributed where dessert and appetizer wines are made and handled in California and other parts of the United States and in Australia. Viable cultures have also been obtained from samples of Spanish sherry and Italian vermouth.

Common names: In California, the bacterium is commonly referred to as the "hair bacillus" and, less frequently, as the "cottony bacillus," "cottony mold," or "Fresno mold."

Distinguishing Characteristics

Lactobacillus trichodes may be distinguished from all other lactobacilli because it has been found only in appetizer and dessert grape wines containing 20 per cent of alcohol by volume; regularly grows as a mass of tangled chains of cells and long filaments forming a flocculent sediment in the depths of these wines leaving the supernatant clear; requires yeast autolysate for its growth and must be trained to grow well in the absence of alcohol, although alcohol is apparently not required for the nutrition of the organism.

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THE TAXONOMY OF LACTOBACILLUS HILGARDII AND RELATED HETEROFERMENTATIVE LACTOBACILLI[1]

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SOME heterofermentative bacteria of the genus *Lactobacillus* differ from the better-known species in that they decompose fewer organic compounds, are more difficult to cultivate, and grow more slowly. Pederson (1938)[5] has referred to these lactobacilli as the "inactive" group. Species which may be included in this group are *Betabacterium caucasicum* Orla-Jensen (1919), *Lactobacillus fructivorans* Charlton, Nelson, and Werkman (1934), and *Lactobacillus hilgardii* Douglas and Cruess (1936). Certain strains of other species reported by Pederson (1938) and the two groups described by Fornachon (1943) as *Lactobacillus* Type I (named *Lactobacillus trichodes* by Fornachon, Douglas, and Vaughn, 1949) and *Lactobacillus* Type II also belong to the inactive group. Their taxonomy is confused.

There are some obvious reasons for the taxonomic confusion. The inactive group as a whole is not well known, either because the bacteria are not prevalent in nature or because their more exacting growth characteristics do not favor isolation from sources containing other lactobacilli having less fastidious requirements. Furthermore, the original descriptions of some of the species are not adequate to allow for comparison with previously described species. Regardless of the manner in which the taxonomy of this "inactive" group is finally treated, it is fundamental that the individual species comprising the group must have adequate descriptions. The following information is presented to establish the identity of *Lactobacillus hilgardii,* for which an adequate description is lacking. (See Pederson, 1939, 1948.) With this identity established, the taxonomy of the "inactive" group of lactobacilli is discussed.

CHARACTERISTICS OF LACTOBACILLUS HILGARDII

Since the original type culture of *Lactobacillus hilgardii* had been lost, it was necessary to isolate new cultures similar to or identical with the original strain. The new isolates were obtained from California table wines. The ten cultures are identical with or closely resemble the type species, as will be seen in the following description.

The new isolates of *Lactobacillus hilgardii* were compared with the type culture of *L. fructivorans,* originally supplied by Pederson; two strains of *Betabacterium caucasicum,* one isolated by Vaughn and one received from

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Tittsler; five new isolates of *L. trichodes;* and ten cultures of *L. brevis.*[6] The cultures of the last two species were isolated from California wines. In addition, comparisons were also made with 30 cultures of *L. brevis* obtained from fermenting olive brines.

NEW DESCRIPTION OF LACTOBACILLUS HILGARDII (DOUGLAS AND CRUESS, 1936)

Morphology (at 30° C)

Form and arrangement: Rods with rounded ends which occur singly, in short chains and frequently in long filaments. Individual cells in stained preparations measure from 0.5 to 0.8 by 2.0 to 4.0 microns. Individual filaments may measure as much as 15 or more microns in length.

Spore formation: Spore formation has not been observed.

Motility: Neither motile cells nor flagella have been observed.

Staining reactions: The cells are Gram-positive in young cultures. In older cultures the cells may become Gram-negative and show granulation.

Cultural Characteristics (at 30° C)

Nutrient agar slant: Growth, if any, faint.

Tryptone yeast extract glucose agar, tryptone tomato juice glucose agar, or yeast infusion glucose agar slants: Growth moderate, raised, hyaline to white and cream-colored with entire edges.

Nutrient broth. Growth, if any, faint.

Tryptone yeast extract glucose broth, tryptone tomato juice glucose broth, or yeast infusion glucose broth: Moderate turbidity is formed in 3 to 4 days, which clears after a few more days and leaves a granular, flocculent sediment. No pellicle or ring of growth is observed.

Gelatine stab (at 20° C) : Growth, if any, faint. No liquefaction after 60 days' incubation.

Litmus milk: No change.

Biochemical Characteristics (at 30° C)

Catalase: This enzyme is not produced.

Fermentation of glucose: The principal end-products are inactive lactic acid, acetic acid, carbon dioxide, ethyl alcohol, and glycerol.

Fermentation of fructose: The fermentation of fructose is characterized by the formation of mannitol in addition to the products mentioned for glucose.

Fermentation of d-*xylose:* The end-products of *d*-xylose fermentation are inactive lactic acid and acetic acid.

Utilization of carbohydrates: Only *d*-xylose and fructose are consistently fermented in suitable basal media (tryptone or yeast infusion broths) adjusted to pH 6.8 to 7.0. The results with glucose and *l*-malic acid are erratic. All other sugars, alcohols, and organic acids tested in the same media with the same initial pH range are not attacked. (The basal media and substrate solu-

[6] The assistance of Mr. J. Richard Gililland in isolating some of these cultures is gratefully acknowledged. We also express our indebtedness to Dr. Carl S. Pederson and Dr. Ralph P. Tittsler for cultures, and to Dr. R. E. Buchanan for assistance with nomenclature.

tions were sterilized separately and mixed aseptically just prior to inoculation, or mixed and sterilized by filtration.)

When, however, the same compounds are tested in the same basal media adjusted to an initial pH in the range 4.5 to 5.5, *d*-xylose, glucose, fructose, galactose, maltose, lactose, sucrose, and raffinose are utilized, as indicated by significant acid production from these substrates. In addition, citric and malic acids are always decomposed. Utilization of other sugars, alcohols, glucosides, or organic acids tested was not detected, which is in agreement with the work of Fornachon, Douglas, and Vaughn (1940).

Neither indole nor hydrogen sulfide were produced, nor was nitrate reduced in any of the media used, although glucose was added to insure growth of the cultures.

Requirements for Growth

Oxygen relations: Facultative. Initial isolation and culture is facilitated by the presence of carbon dioxide.

Temperature requirements: The optimum temperature for maximum acid formation from glucose in 7 days ranges from 28° to 34° C. The minimum temperature for growth in glucose media is approximately 15° and the maximum temperature is in the range of 40° to 43°.

pH range: The optimum initial pH value is variable. For maximum cell production and decomposition of glucose by growing cultures, the optimum initial pH range is from 4.5 to 5.5. For maximum decomposition of citric and malic acids, the optimum initial pH range is between 4.5 and 5.0. Growth has been observed in media with initial pH values of 3.8 and 8.0.

Alcohol tolerance: The limiting concentration of alcohol for growth of cultures in wine is between 15 and 18 per cent by volume.

Salt tolerance: The limiting concentration of salt (sodium chloride) for growth of cultures in suitable media is between 5 and 6 per cent (grams NaCl per 100 ml of medium).

Suitable media: The best medium for growth and maintenance of cultures of *Lactobacillus hilgardii* is liver infusion broth with liver particles. Cultures grown in this medium and held at 0° C remain viable for at least one year. Liver infusion broth with or without liver particles probably is best for preparation of cultures to be used as inocula. For growth of isolated colonies or mass cultures, liver infusion agar, tryptone glucose agar with tomato juice, or yeast infusion or autolysate glucose agar are satisfactory.

Distinguishing Characteristics

The characteristics of *Lactobacillus hilgardii* which differentiate it from the *d*-xylose, galactose, sucrose, maltose, and *l*-malic acid; of these, *L. fructivorans* ferments only *l*-malic acid (weakly), *Betabacterium caucasicum* only galac-
ferments only *1*-malic acid (weakly), *Betabacterium caucasicum* only galactose (weakly), and *L. trichodes* only sucrose and maltose (both weakly). *L. hilgardii* does not ferment *l*-arabinose or mannose, whereas *L. brevis* ferments them strongly and *B. caucasicum* ferments mannose weakly. Morphological differences also are useful for distinguishing *L. hilgardii* from *L. brevis* and *B. caucasicum*. It will be noted that *L. hilgardii* and *Lactobacillus* Type II are similar. We believe that they probably are identical species.

Table 1

DIFFERENTIATION OF "INACTIVE" GAS-FORMING (HETEROFERMENTATIVE) SPECIES OF LACTOBACILLUS

Species and number of cultures	Morphology		Filamentation	Optimum conditions for glucose fermentation		Fermentation* of														Alcohol tolerance,† per cent
	Cell size, μ	Cell arrangement		Temperature range, °C	Initial pH range	*d*-Xylose	*l*-Arabinose	Glucose	Fructose	Mannose	Galactose	Sucrose	Maltose	Lactose	Raffinose	Glycerol	Mannitol	*l*-Malic acid	Citric acid	
L. hilgardii, 10 cultures	0.5–0.8 × 2.0–4.0	Single and short chains	Frequent	30–35	4.5–5.5	++	–	±	++	–	+	+	+	±	±	–	–	++	±	15–18
L. fructivorans, 1 culture	0.5–0.8 × 1.5–4.0	Chains of rods	Frequent	25–30	4.5–5.5	–	–	+	++	–	–	–	–	–	–	–	–	±	–	15
L. trichodes, 5 cultures	0.4–0.6 × 2.0–4.0	Long chains	Marked; intertwining	25–30	4.5–5.5	–	–	++	++	–	–	±	±	–	–	–	–	–	–	>20
Lactobacillus, Type II,‡ 4 cultures	0.6–0.8 × 2.0–4.0	Single, pairs, and chains	Frequent	30–35	4.5–5.5	++	–	++	++	–	+	+	+	–	–	–	–	+	±	18–20
Betabacterium caucasicum, 2 cultures	0.7–1.0 × 2.0–4.0	Single rods	Frequent	25–30	5.0–7.0	–	++	++	++	±	++	–	–	–	–	–	–	–	–	15
L. brevis, 40 cultures	0.7–1.0 × 2.0–4.0	Single and pairs	Occasional	30–35	4.5–7.5	++	++	++	++	+	++	+	++	+	+	±	±	++	++	15–18

* –, No activity; ±, weak activity; + moderate activity; ++, maximum activity. All fermentations were conducted at the optimum initial pH range for each culture.
† Alcohol tolerance (per cent by volume) on basis of growth in wine with 1 ml yeast autolysate per 100 ml of wine.
‡ Data for *Lactobacillus* Type II taken from Fornachon (1943).

TAXONOMIC RELATIONSHIPS OF SPECIES OF THE "INACTIVE" GROUP

The description of *Lactobacillus hilgardii* makes it possible to consider the relationships of the "inactive" species to each other and to other species which have been described in the literature. Pederson (1938) considered *Betabacterium caucasicum, L. fructivorans,* and "a culture labeled *Bacterium mannitopeum*" as resembling the *L. brevis* group because the cultures had similar optimum temperatures and fermented *l*-arabinose. Although Pederson indicated the need for further study, this raises the question of whether *L. hilgardii* also resembles the *L. brevis* group and whether the inactive cultures investigated here are merely strains of *L. brevis*. Moreover, Orla-Jensen *et al.* (1947) have recently expressed their willingness to make the name *Betabacterium caucasicum* a synonym of *Betabacterium pentoaceticum*. We contend, however, that there are several well-defined species of "inactive" heterofermentative lactobacilli.

The fermentation of pentoses and optimum temperature relations are used as primary characters for separation of the heterofermentative species of *Lactobacillus*. If these are valid criteria for taxonomic differentiation, as maintained by Orla-Jensen (1919, 1943) and Pederson (1938, 1939, 1948), then none of the "inactive" species are more closely related to one another or to *L. brevis* than are the other commonly recognized species *L. buchneri, L. fermenti,* and *L. pastorianus* to one another or to *L. brevis*.

It is true that the "inactive" species do resemble *Lactobacillus brevis* when optimum and maximum temperature relations are considered. (They all have optimum temperatures ranging between 25° and 35° C and maximum temperatures of about 40° C or even less.) However, separation of the "inactive" species from *L. brevis* is not difficult when pentose and other carbohydrate fermentations are considered, as has been shown in table 1. Other criteria which serve to separate the "inactive" species from *L. brevis* include differences in rate of growth, type of growth, growth requirements, cell morphology, and the effect of pH on fermentation (table 1).

The general growth characteristics of *Lactobacillus hilgardii, L. fructivorans, L. trichodes,* and *Betabacterium caucasicum* as compared with *L. brevis* are important for differential purposes. The "inactive" species all grow slowly even under optimum conditions. Visible signs of growth appear only after from 4 or 5 days to as long as 2 weeks. These species also tend to produce a flocculent sediment composed of chains of cells and filaments in the depths of liquid cultures, so that the supernatant liquid is left permanently clear or almost clear for some time. Cultures of *L. brevis,* on the other hand, produce abundant growth in 2 days or less; quickly grow throughout the liquid; and produce a minimum of filaments.

The "inactive" species also are much more exacting in their requirements for growth. Initial isolation and culture is facilitated by the presence of carbon dioxide; otherwise purification and maintenance of cultures is difficult. Growth of all the "inactive" species is stimulated markedly by the addition of tomato juice or yeast autolysate to the culture media; yeast autolysate is

required for growth of *Lactobacillus trichodes*. *L. brevis* also responds to these conditions but less markedly, and the conditions are not mandatory.

The effect of the initial pH value of the medium on growth and fermentative activity, if properly assessed, also serves to differentiate the "inactive" species from the *Lactobacillus brevis* group. This effect has been previously stressed (Fornachon, Douglas, and Vaughn, 1940).

Lactobacillus hilgardii, *L. fructivorans*, and *L. trichodes* bear some physiological resemblance to *Bacterium gracile* (*L. gracilis*) Müller-Thurgau (1908). However, on the basis of morphological study of available cultures, Charlton, Nelson, and Werkman (1934) concluded that *Bacterium gracile* was a member of the genus *Leuconostoc* rather than *Lactobacillus*.[7] Pederson (1939) expressed a similar opinion. If this is true, there is obviously no possibility that *L. hilgardii*, *L. fructivorans*, or *L. trichodes* corresponds with *Bacterium gracile*. We believe therefore that *L. hilgardii*,[8] *L. fructivorans*, *L. trichodes*, and *Betabacterium caucasicum* are well-defined species of heterofermentative lactobacilli.

We propose to transfer the species *Betabacterium caucasicum* to the genus *Lactobacillus* because the latter is more widely accepted as the genus name for the heterofermentative lactobacilli than *Betabacterium*, in this country at least. The specific name *caucasicus* is preëmpted for the type species of *Lactobacillus*. Hence the name *Lactobacillus desidiosus* (from Latin *desidiosus*, inactive, indolent) is proposed to replace the name *Betabacterium caucasicum*.

Synonyms of *Lactobacillus desidiosus* are *Betabacterium caucasicum* Orla-Jensen, The Lactic Acid Bacteria, 1919, 175; and *Betabacterium pentoaceticum* Orla-Jensen, Orla-Jensen and Kjaer, Antonie van Leeuwenhoek, *12*, 1947, 112; the latter in part only. *Lactobacillus pentoaceticus* Fred, Peterson, and Davenport, J. Biol. Chem., *39*, 1919, 358; Peterson and Fred, J. Biol. Chem., *41*, 1920, 431; and Fred, Peterson, and Anderson, J. Biol. Chem., *48*, 1921, 385, is described as actively fermenting both arabinose and xylose. It is therefore considered a synonym of *Lactobacillus brevis* (Orla-Jensen) Bergey *et al.*, by Pederson.

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[7] Credence for this conclusion is strengthened by comparison of photographs published by Müller-Thurgau (1908) and Müller-Thurgau and Osterwalder (1913, 1918) with those of Charlton, Nelson, and Werkman (1934).

[8] It has been claimed through an error (Cruess, 1943) that Vaughn and Douglas considered *L. hilgardii* to be very similar to *L. plantarum*. The fallacy has persisted (Cruess, 1947; Olsen, 1948). The heterofermentative nature of *L. hilgardii* was obvious in the first incomplete description and in Fornachon, Douglas, and Vaughn (1940).

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